

The effects of exposure of susceptible alpacas to alpacas persistently infected with bovine viral diarrhea virus

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Abstract — Reports of bovine viral diarrhea virus (BVDV) infections in alpacas have been increasing in recent years but much is still unknown about the mechanisms of disease in this species. This report characterizes the transmission of BVDV from persistently infected (PI) alpacas to BVDV naïve alpacas, documents shedding patterns, and characterizes the disease effects in both PI and transiently infected alpacas. Two PI alpacas shed BVDV Type 1b virus in most body fluids, and commonly available diagnostic tests verified their status. Bovine viral diarrhea virus Type 1b transient infections produced only mild signs of disease in BVDV naïve alpacas. Viremia was detected in whole blood, but viral shedding during the acute phase was not detected and antibody appeared to be protective upon re-exposure to the virus.

Résumé — Effets de l'exposition d'alpacas susceptibles à des alpacas infectés de manière persistante par le virus de la diarrhée virale bovine. Les rapports d'infection par le virus de la diarrhée virale bovine (VBVD) chez les alpacas ont augmenté au cours des dernières années mais nous en savons encore peu à propos des mécanismes de la maladie chez cette espèce. Ce rapport caractérise la transmission du VBVD d'alpacas avec une infection persistante (IP) à des alpacas naïfs pour le VBVD, documente les schémas d'excrétion et caractérise les effets de la maladie chez les alpacas avec une infection persistante et chez ceux avec une infection transitoire. Deux alpacas IP ont excrété le virus de type 1b de la BVD dans la plupart des liquides organiques et des tests de diagnostic couramment disponibles ont vérifié leur statut. Les infections transitoires par le type 1b du virus de la diarrhée virale bovine ont produit seulement des signes légers de maladie chez les alpacas naïfs pour le VBVD. Une virémie a été détectée dans le sang entier, mais l'excrétion virale durant la phase aiguë n'a pas été détectée et les anticorps ont semblé être protecteurs lors d'une nouvelle exposition au virus.

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Introduction

Bovine viral diarrhea viruses (BVDV-1 and BVDV-2) are members of the pestivirus genus that includes Border Disease virus of sheep and Classical Swine Fever (Hog Cholera virus) in swine (1,2). Bovine viral diarrhea virus was first recognized as a pathogen of cattle in 1946; this virus also infects other even-toed ungulates with varying degrees of disease severity (3,4). The first report of BVDV antibodies in camelids was published in 1983, and prior to 2005 there were only sporadic

reports of BVDV in llamas and alpacas (5–9). Beginning in 2005, reports of BVDV-infected alpacas and persistent infections began emerging, followed more recently by seroprevalence, genotyping, and phylogenetic analysis of the virus in alpacas (10–17). A recent increase in reported cases of BVDV in alpacas likely involves variations in virus strain, animal management practices, as well as increased awareness and testing by producers.

Transiently infected (TI) and persistently infected (PI) cattle can transmit BVDV in various body fluids, with the primary

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portal of infection being the oronasal mucosa (18–20). Both TI and PI cattle are sources of infection for other cattle. Transiently infected cattle typically shed BVDV for only a few days, whereas PI cattle are believed to either continuously shed large quantities of virus or inconsistently shed as evidenced by intermittent viremia (21–24). Similar shedding and transmission patterns are suspected in PI and TI alpacas, but have not been reported. Based on identification of the virus in tissues such as salivary glands, kidneys, gastrointestinal tract, lungs, testicles, and prostate, alpacas may shed virus in a range of body fluids similar to cattle (11,12,14,16).

Cattle become persistently infected with BVDV if the fetus is exposed to the virus before the immune system is fully developed, resulting in immune tolerance whereby the fetus does not recognize the virus as foreign (18). Fetal infection can occur if the dam develops a transient infection or is herself a PI animal (25). Persistently infected calves typically show signs of ill-thrift and chronic illnesses; however, they can also appear clinically normal (26). Similar evaluations have not been well described in PI alpacas as most have been diagnosed immediately prior to death or euthanized following diagnosis (11,12,14,16).

Research in BVDV PI alpacas is lacking in part due to the relative infrequency of the condition, the long gestation period of alpacas (approximately 11 mo), and the likelihood that most crias suspected of being PIs are euthanized or not reported. The present study was designed to verify the infection status of alpacas persistently infected with BVDV, to evaluate transmission of BVDV from PI alpacas to naïve alpacas, to characterize shedding patterns in transiently infected animals, and to describe the effects of BVDV infections in PI and TI alpacas.

Materials and methods

Animals

Two alpaca crias from a single farm (5-month-old intact male “WSU-S”; 6-month-old female “WSU-D”) were donated to the Washington State University Veterinary Teaching Hospital. The 2 crias were verified as being persistently infected with BVDV based on positive BVDV polymerase chain reaction (PCR) and virus isolation (VI), and negative BVDV serum neutralization (SN) on monthly checks starting at 2 to 3 mo of age as described below. Sequencing identified the strain as BVDV Type 1b. Six clinically healthy, intact male alpacas (5- to 6-months-old) were used as BVDV-negative infection controls; they were verified as BVDV negative based on PCR and SN as described below. The cutoff for negative SN antibody concentration was 1:4.

The studies were conducted in accordance with the Washington State University Institutional Animal Care and Use Committee. The limited number of experimental animals was chosen and justified based on humanitarian grounds since BVDV is known to induce immunosuppression and cause synergistic interactions with other diseases in other species.

Experimental exposure

Two experimental exposure periods occurred over a period of 32 wk. The 6 BVDV negative alpacas were exposed to the PI alpacas during weeks 1 to 4 and weeks 18 to 19. All alpacas

were maintained in enclosed isolation facilities and both exposure periods occurred in the PI alpaca isolation facility. Three BVDV negative alpacas (1A-1C) and the 2 PI alpacas (WSU-S, WSU-D) were placed together in an 8 × 10-ft pen (pen 1) and the other 3 alpacas (2D-2F) were placed in a similar sized adjacent pen (pen 2) separated by a 6-ft high chain link fence. Alpacas in both pens were provided with a free choice of grass and alfalfa hay and a commercial pelleted alpaca ration with trace minerals, and had separate water sources. Alpacas in pen 1 were also given a commercial calf starter ration due to the low body condition scores (BCS) of the PIs (BCS 3/10). Pens were cleaned daily, with pen 2 being cleaned prior to pen 1. Vital parameters (temperature, pulse, and respiration rates), and gastrointestinal compartment 1 contraction rates were obtained twice daily on all animals (pen 2 prior to pen 1) during weeks 1 to 3. The BCS of all alpacas were measured on a weekly basis as described by Fowler (27).

After the initial 4-week exposure period, the 6 non-PI alpacas were removed to a second enclosed isolation facility and maintained as separate groups in order to monitor serum antibody concentrations. At the start of week 18, the 6 non-PI alpacas were re-introduced to the remaining PI alpaca (WSU-S) in the original isolation facility and pen configuration as described above. Vital parameters were obtained daily during week 18.

Sampling

Blood, nasal, and oral swabs, and fecal samples were obtained from the 6 non-PI alpacas every 3 d during weeks 1 to 3 of the combined housing. Blood samples were then obtained weekly for weeks 4 to 8 and every 2 wk for weeks 9 to 17. During the re-exposure trial (weeks 18 to 19), blood, nasal, and oral swabs were collected from the 6 non-PI alpacas once daily for week 18, every third day for week 19, then at weeks 21 and 32. Similar body fluid samples were obtained from the 2 PI alpacas once a month to monitor BVDV persistent infection status. Samples of blood (12 mL) were aseptically obtained from the jugular vein and divided between ethylenediaminetetraacetic acid (EDTA), acid citrate dextrose (ACD), and serum blood collection tubes (BD Vacutainer Blood Collection Tubes; Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA). Individual nasal and oral swabs were collected from the lateral wall of the nasopharynx and buccal surface of the oral cavity. Swabs were placed in individual viral collection vials (BD Universal Viral Transport Kit 220222, Becton, Dickinson and Company). Fecal samples were obtained by digital collection from the rectum and placed in sterile plastic specimen bags. When possible, free-catch urine samples were collected in sterile specimen cups.

Skin samples were obtained from the PI alpacas by placing a sterile hemostat 0.5 cm from the end of the pinna and using a #10 scalpel blade to excise the ear tip. The tissue samples were placed in individual serum blood collection tubes. Hemostasis was maintained for 5 min before removal of the hemostat. Samples not immediately processed were stored at –20°C until analysis. After euthanasia of the PI alpaca, WSU-D, a full necropsy was performed with a complete complement of tissues collected.

Hematologic analyses

Blood samples collected in EDTA blood collection tubes were used to determine hematologic profiles. The total leukocyte count, red blood cell count, hemoglobin concentration, platelet count, and mean platelet volume were determined with an automatic cell counter (Advia 120, Siemens Corporation, New York, New York, USA). Blood smears were prepared from the EDTA-treated blood and stained with Wright-Giemsa stain, and the differential leukocyte counts were completed via microscopic examination. Hematocrit, fibrinogen, and plasma protein concentrations were manually determined following standard laboratory protocols. Fibrinogen and plasma protein concentrations were measured using a refractometer. Blood collected in serum collection tubes was allowed to clot and the serum separated and analyzed within 2 h of collection. Analyses for serum concentrations of total protein, albumin, and globulins were performed using an automated chemistry analyzer (Dimension Xpand Plus System; Siemens Corporation).

Virus isolation, PCR, and sequencing

Virus isolation from whole blood, oral and nasal swabs, and tissue samples was attempted in bovine turbinate (BT) cells free of BVDV (28). The inoculated cells were passaged 3 times at 3-day intervals and then the supernatant fluid was analyzed for the presence of BVDV RNA by PCR (29). Bovine viral diarrhea virus RNA was extracted from the supernatants of virus-infected cells or directly from EDTA blood using the Magmax -96 Blood RNA Isolation Kit (Ambion, Austin, Texas, USA). Real Time Taqman PCR to detect BVDV was performed using an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, California, USA) and the ABI AgPath-ID BVDV Reagent Kit.

The basis of the phylogenetic analysis was comparison of 5' untranslated region (UTR) sequences generated by cycle sequencing of PCR amplicons as described previously (30). Total RNA was prepared from infected cells using QIAamp Viral RNA Mini Kit (Qiagen Sciences, Valencia, California, USA) as per the manufacturer's directions. A 10-mL aliquot of the total RNA was used per reverse transcriptase PCR reaction. The PCR primers, based on sequences from the 5' UTR have been described previously (31). The PCR products were quantitated using the Pico Green assay for double stranded (ds) DNA (Invitrogen Corporation, Carlsbad, California, USA). The appropriate quantity of dsDNA PCR product was labeled in both directions using Big Dye terminator chemistries (Applied Biosystems) according to the manufacturer's instructions. The labeled products were sequenced using an ABI 3100 genetic analyzer (Applied Biosystems). Sequences were aligned and compared based on the Higgins-Sharp algorithm (CLUSTAL4) using the MacDNASIS program (Hitachi Software, San Bruno, California, USA). This program takes as input a dendrogram produced by applying the unweighted pair group method using arithmetic average (UPGMA) to a matrix of similarity scores for all the aligned sequences (32). The similarity scores are calculated as the number of exactly matched residues in a Wilbur and Lipman alignment minus a fixed penalty for every gap (33). Segregation into subgenotypes was based on

Table 1. Monoclonal antibodies (mAb) used to identify cell populations in whole blood collected from male alpacas exposed to BVDV PI alpacas

mAb	Isotype	Specificity	Cell phenotype
LH9A	IgM	Activation marker	
LH41A	IgG2a		B-cells
FITC-Ig	IgG	sIg	B-cells
GB45A	IgG1	WC1	$\gamma\delta$ T-cell subset
LT10A ^a	IgG2a	CD2, CD6	$\alpha\beta$ T-cell subset
LT97A ^a	IgG2b	CD3	Mature T-cells ($\alpha\beta$ and $\gamma\delta$)
GC50A1	IgM	CD4	T cells (helper/inducer)
LT3A1 ^a	IgG1	CD5	Mature T-cells ($\alpha\beta$ and $\gamma\delta$)
LT5A	IgG2a	CD8	T-cells (cytotoxic/suppressor)
DH59B1A	IgG1	CD172a	Monocytes

^a Predicted specificity based on pattern similarity with other species (37).

comparison to BVDV-1 subgenotypes (34,35) and BVDV-2 subgenotypes (36).

Antigen ELISA

Serum and skin samples were tested for BVDV antigen using the IDEXX HerdChek Bovine Virus Diarrhea Antigen Test Kit (IDEXX Laboratories, Westbrook, Maine, USA). Briefly, 100 μ L of serum or supernatant was placed in pre-wetted wells, along with positive and negative standards, and in-house reference control sera. Testing was done according to IDEXX protocols. Samples with standard to positive (S/P) ratios of < 0.20 were classified as "BVDV-Negative." Samples with S/P ratios of 0.20 to 0.39 were classified as "Suspect," and samples with an S/P ratio of > 0.39 were classified as "BVDV-Positive." Suspect and positive reactors were assayed twice, first using the standard working detector reagent, and then using a "modified" working detector reagent to check for non-specific reactivity. Optical densities were measured using a Bio-Tek EL808 Ultra Microplate Reader (Bio-Tek Instruments, Winooski, Vermont, USA), and calculations were made with KC-4 Bio-Tek software.

Serum neutralization

Serum was analyzed for BVDV type 1-specific antibodies using the serum neutralization (SN) assay (28). Alpaca serum was diluted in 2-fold serial dilutions using minimal essential medium (MEM) plus antibiotics [2.5 μ g/mL Fungizone (JR Scientific, Woodland, California, USA), 100 International Units (IU)/mL penicillin G, 100 IU/mL streptomycin sulfate, and 50 μ g/mL gentamicin] beginning at a screening dilution of 1:4. Briefly, 50 μ L of MEM plus was added to wells of a flat-bottom 96-well microtiter plate and 50 μ L alpaca serum and known positive control serum were added to the appropriate wells. Two-fold serial dilutions were performed with an end volume of 50 μ L in each well. The Singer strain of cytopathic BVDV (Type 1a) was used as the challenge virus at 100–1000 TCID₅₀ per 50 μ L. The plate was incubated at room temperature for 1 h after which 50 μ L of a predetermined BT cell (BVDV-free) concentration were added to each well as the indicator cell type followed by incubation in a 10% humidified, 5% CO₂ incubator at 37°C. At 72 h, the plate was read for serum neutralization of viral cytopathic effect. Serum antibody titers were expressed as the reciprocal of the highest dilution of serum providing 100% cell

Table 2. Monoclonal antibody (mAb) combinations used for two-color flow cytometric analyses

Well number	mAb Combination	Cell phenotype identified
1	Control	
2	LT97A, DH59B1A	Mature T-cells ($\alpha\beta$ and $\gamma\delta$), Monocytes
3	LT97A, LH41A	Mature T-cells ($\alpha\beta$ and $\gamma\delta$), B-cells
4	LT97A, LH9A	Mature T-cells ($\alpha\beta$ and $\gamma\delta$), Activation marker
5	LT97A, GB45A	Mature T-cells ($\alpha\beta$ and $\gamma\delta$), $\gamma\delta$ T-cell subset
6	LT97A, FITC-Ig	Mature T-cells ($\alpha\beta$ and $\gamma\delta$), B-cells
7	LT10A, LT3A1	$\alpha\beta$ T-cells subset, Mature T-cells ($\alpha\beta$ and $\gamma\delta$)
8	LT5A, GC50A1	T-cell (cytotoxic/suppressor), T-cell (helper/inducer)

Table 3. Diagnostic test results for BVDV PI alpacas (WSU-S and WSU-D)

Month ^a	PI Alpaca	Sample	Diagnostic test ^b			
			PCR	SN	VI	AgELISA
1	WSU-D, WSU-S	blood	+	—		
2	WSU-D, WSU-S	blood	+	—	+	+
3	WSU-D, WSU-S	blood	+	—	+	+
		oral & nasal	+		+	
4	WSU-D, WSU-S	blood	+	—		
5	WSU-D, WSU-S	blood	+	—		
		ear notch				+
6	WSU-D	feces, urine	+		+	
	WSU-D, WSU-S	blood	+	—		
7	WSU-S	blood	+	—		+
8	WSU-S	blood	+	—		
		oral & nasal	+		+	
9	WSU-S	blood	+	—		
10	WSU-S	blood	+	—		
11	WSU-S	blood	+	—		
		oral & nasal	—		—	
12	WSU-S	blood	+	—	+	
		urine, oral & nasal	+		+	

PCR — polymerase chain reaction; SN — serum neutralization; VI — virus isolation; AgELISA — antigen capture enzyme-linked immunosorbent assay.

^a Month indicates the period after which the alpacas arrived at the WSU VTH.

^b Diagnostic test results lacking (+) or (—) indicates that tests were not performed on the specified samples. PCR and virus isolation were performed on whole blood and oral and nasal secretions. AgELISA tests were performed on serum and ear skin samples.

protection. Seroconversion was defined as the demonstration of detectable antibody following an earlier negative result ($< 1:4$).

Flow cytometry

Mouse derived monoclonal antibodies (mAbs) (Table 1) were obtained from the Washington State University (WSU) Monoclonal Antibody Center (Pullman, Washington, USA) with the exception of the fluorescein conjugated goat anti-camelid IgG (Triple J Farms, Richland, Washington, USA). Monoclonal antibodies GC50A1 and GB45A were developed against goat leukocytes and the remainder were developed against llama leukocytes (37). The mAbs were used in dual fluorescence combinations which allowed for discrimination between the populations. The pan T (LT97A) mAb was used in combination with DH59B1A to identify T-cells and monocytes. The pan T mAb was used in combination with LH41A or anti-camelid IgG to identify B-cells. The mAbs specific for CD4+ (GC50A1), CD8+ (LT5A) and $\gamma\delta$ (GB45A) T-cells were used to identify the lymphocyte subsets.

Whole blood was collected in ACD, and peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation using Accu-Paque (density, 1.086 g/mL; Accurate Chemical & Scientific Corps., Westbury, New York, USA). The buffy coat layer was collected from the cell-plasma interface and

washed 3 times in phosphate-buffered saline (PBS) containing 20% ACD to remove excess platelets. Cells were resuspended in PBS/ACD. Concentration of the cell suspension was determined using a hemocytometer.

One million cells were added to each well of a 96-well v-bottom microtiter plate containing 2 mAbs (Table 2), (15 $\mu\text{g/mL}$ in 50 μL) in 100 μL of dilution buffer [PBS containing 0.5% horse serum (Invitrogen/GIBCO, Gaithersburg, MD, USA), 20% ACD and 0.02% azide]. Cells were incubated for 15 min at 4°C and then washed 3 times in dilution buffer. Following washing, cells were incubated for 15 min at 4°C with 50 μL of isotype-specific fluorescein or phycoerythrin conjugated goat anti-mouse immunoglobulin (Invitrogen/Caltag, Burlingame, California, USA). The fluorescein-conjugated anti-camelid IgG was added at the same time as the second step reagents. A control was incubated with second step reagents only. The cells were again washed 2 times with dilution buffer without horse serum, resuspended in 2% formaldehyde in PBS (200 $\mu\text{L/well}$) and stored at 4°C in the dark until data could be collected and analyzed. A FACS Calibur flow cytometer with Cell Quest software (Becton Dickinson Immunocytometry Systems, San Jose, California, USA) collected the cell population data and the FCS Express software (De Novo Software, Thornton, Ontario) was used for analysis.

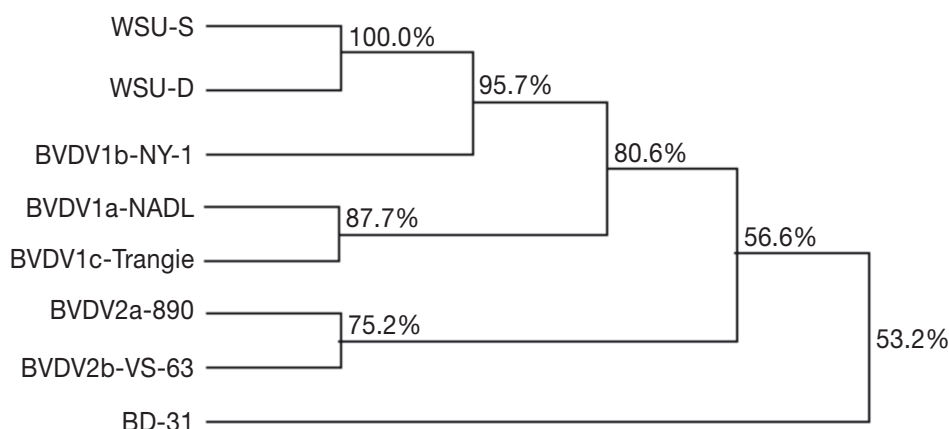


Figure 1. Phylogenetic analysis and relatedness of the BVDV persistently infected alpaca crias WSU-S and WSU-D to other known BVDV strains. The 5' UTR genome section was used in the analysis (30).

Flow cytometry results were evaluated and compared over time and relative to normal values established by the WSU Monoclonal Antibody Center. The normal values were established from 33 apparently healthy llamas ($n = 13$) and alpacas ($n = 20$) between the ages of 1 and 11 y. A combination of llamas and alpaca values was used since the Monoclonal Antibody Center determined that there was no difference in values.

Histopathology and immunohistochemistry

Tissue samples for histopathologic examination were collected into 10% buffered formalin, processed overnight in an automatic processor (Tissue-Tek VIP, Sakura Finetek Japan, Tokyo, Japan), embedded in paraffin, and 4- μ m thick sections were placed onto glass slides. The sections were de-paraffinized with xylene and stained with hematoxylin and eosin. Immunohistochemical (IHC) detection of BVDV antigen was performed on sections of formalin-fixed, paraffin-embedded tissues using monoclonal antibody (mAb) 15c5 [IDEXX MoAB 15c-5 (anti-BVDV EO), IDEXX Laboratories] at a 1:1000 dilution in a streptavidin-biotin-immunoperoxidase technique with diaminobenzidine as a chromogen (LSAB™2 Kit, Dako North America, Carpinteria, California, USA) (38). Replicate sections were incubated with isotype-matched, irrelevant monoclonal antibody as a negative control. Positive control tissue included skin from a BVDV infected bovine calf.

Statistical analysis

Data were entered into Excel spreadsheets (Microsoft, Bellevue, Washington, USA) and analyzed with simple statistics to calculate mean and standard deviations. A paired Student *t*-test was used to compare pre- and post-exposure flow cytometry results. A value of $P < 0.05$ was considered significant.

Results

Verification of PI status

Persistently infected status was verified monthly by positive PCR and negative SN test results (Table 3) and both WSU-D and WSU-S retained their BVDV PI status. Antigen ELISA was

performed intermittently on both serum and ear notch skin samples and was consistently positive in both animals. Viral shedding was verified by PCR and VI testing of periodic oral and nasal swabs. Swabs from WSU-D were consistently positive, whereas swabs from WSU-S were positive except for those collected at 11 mo. Urine samples were PCR and VI positive from both PI alpacas and feces were positive from WSU-D. Viral genomic sequence comparisons were made with the 5' UTR section and sequencing most closely matched that of BVDV-1b (Figure 1).

PCR and serology

Alpacas 1A and 1C housed in direct contact and alpaca 2F housed in fence-line contact with the PI alpacas became PCR positive for BVDV on the EDTA blood samples on day 5; all others were positive on day 8 of exposure. All 6 animals remained PCR positive through day 18. Alpacas 1A and 1C seroconverted from seronegative ($< 1:4$) to seropositive (1:4) for BVDV Type 1 by day 18; alpacas 2D, 2E and 2F by day 21, and alpaca 1B by day 27. Serum antibody concentrations continued increasing after separation from the PI alpacas before peaking at 1:128 to $> 1:512$ (between weeks 8 and 11) and remaining relatively constant until the second exposure at week 18. Serum antibodies for BVDV type 2 were not detected in any samples. Oral and nasal swabs collected from all 6 transiently infected alpacas during the viremic stage were BVDV negative by VI and PCR.

During the second exposure, none of the 6 previously exposed alpacas had detectable virus by PCR performed on EDTA blood samples and no significant changes occurred in serum titer.

Hematologic analyses

Complete blood cell counts in the 6 TI alpacas revealed only mild changes with all white blood cell parameters and fibrinogen levels remaining within normal limits. Four alpacas (1B, 1C, 2D, 2E) developed mildly decreased hematocrits (23% to 26%; reference range: 27% to 45%) 3 to 7 d after becoming PCR positive, and the decreased hematocrit values persisted for 1 to 3 wk. No other abnormalities were detected.

Table 4. Flow cytometric results from BVDV persistently infected alpacas obtained at 5, 7, 9, and 11 months of age. Mean values and ranges are from both alpacas at 7 months of age. Results indicate percent positive of labeled cells

Cell phenotype	Normal values ^a		Age of persistently infected alpacas				
	1 to 11 months (<i>n</i> = 33)		5 months (<i>n</i> = 1)	7 months (<i>n</i> = 2)		9 months (<i>n</i> = 1)	11 months (<i>n</i> = 1)
	Mean	Range		Mean	Range		
αβ T Lymphocytes	28	11–47	25	57.5	51–64	31	29
CD4+ T Lymphocytes	21	11–49	22	30	21–39	17	21
CD8+ T Lymphocytes	10	4–28	4	19.5	14–25	12	9
γδ T Lymphocytes	11	4–26	7	3.5	1–6	9	9
B Lymphocytes	42	26–60	54	20	19–21	42	32
Monocytes	19	6–39	8	21	11–31	16	13

^a Normal values from 1- to 11-month-old camelids provided by WSU Monoclonal Antibody Center.

Complete blood cell counts for the 2 PI alpacas were variable. Blood work from alpaca WSU-D revealed consistently low normal leukocyte counts (4000 to 7900/μL; reference range: 8000 to 21 000/μL), neutropenia (443 to 1140/μL; reference range: 1200 to 7200/μL), and anemia (18% to 24%). Alpaca WSU-S experienced intermittent mild anemia (21% to 26%) with no other abnormalities detected.

Physical examination

There were mild changes in physical examination findings. Elevated rectal temperatures (39.3°C to 39.6°C; reference range: 37.2°C to 39.2°C) were detected in 3 animals (1B, 1C, 2D) for 1–3 d beginning on day 11 of exposure to the PI alpacas. Alpacas in both pens developed soft feces for up to 11 d beginning on day 8 of exposure. Two alpacas (1A, 1C) housed in pen 1 developed diarrhea on days 15 and 17, respectively. Excessive serous nasal discharge was present in 2 alpacas (2E, 2F) on days 5 and 6 of exposure, though no coughing or sneezing occurred and the respiratory rate remained within normal limits. No abnormalities were noted during the second exposure period.

Physical examination findings of PI alpacas WSU-D and WSU-S were variable. Alpaca WSU-D experienced intermittent febrile periods with rectal temperatures up to 39.6°C every 3 to 5 d that persisted for 24 to 36 h, and also developed diarrhea for 1 to 2 d during several of the febrile periods. The diarrhea resolved without treatment. Both PI alpacas showed signs of recurrent excessive serous to mucopurulent nasal discharge that were responsive to treatment with ceftiofur hydrochloride (Excenel; Pfizer Animal Health, New York, New York, USA) or florfenicol (Nuflor; Intervet/Schering-Plough Animal Health, Summit, New Jersey, USA). The 2 PI alpacas remained thin (BCS 4/10) and did not increase in weight for the duration of the project when compared to the 6 non-PI alpacas.

Flow cytometry

Whole blood samples from the PI alpacas were analyzed by flow cytometry every 2 mo after arrival (Table 4). Cell population percentages were variable but generally remained within the normal values for juvenile alpacas (WSU Monoclonal Antibody Center). Both alpacas experienced intermittently elevated αβ T-cell populations (64% in WSU-D and 51% in WSU-S; normal range 11% to 47%) at 7 mo of age, with simultaneously decreased B lymphocytes (19% in WSU-D and 21% in WSU-S;

normal range: 26% to 60%) but values returned to normal at the next testing.

Blood from the 6 non-PI alpacas, obtained 4 wk prior to the first exposure, and at week 4, week 17 (immediately prior to second exposure), week 18 (day 5 of re-exposure), and week 19 (day 14 of re-exposure) was analyzed by flow cytometry (Figure 2). The mean values for all cell subsets were within the normal ranges (WSU Monoclonal Antibody Center) prior to exposure to the PI alpacas. When compared using a paired Student *t*-test, week 4 values were significantly different from the pre-exposure values for all cell subsets except for the αβ and γδ T lymphocytes. At week 4 of exposure, B lymphocyte and monocyte populations were low or low normal and the T lymphocyte subsets were increased from the pre-exposure sampling. Over the following weeks, B lymphocytes remained relatively constant slightly below or in the low normal range. Monocytes fluctuated in the low normal range and then decreased significantly again during the second exposure period between week 17 (prior to exposure) and week 18 (day 5 of re-exposure). No significant changes were observed in the other cell populations during the re-exposure period. The αβ T-cell and CD4+ T-cell subset populations remained within the high normal range. The CD8+ T-cell subset decreased by week 17 but remained slightly above the normal mean for the remainder of testing. The γδ T-cell subset showed a more gradual increase and remained within the normal range.

Histopathology and immunohistochemistry

Postmortem examination of WSU-D revealed severe sinusitis and rhinitis with inflammatory tract formation through the frontal sinus, thymic atrophy, and ulcerative gastritis in compartment 3. Histologic examination revealed generalized, moderate to severe lymphocyte depletion from tonsil, thymus, spleen and submandibular, cervical, mesenteric, and inguinal lymph nodes. The liver exhibited rare, multifocal hepatocellular necrosis speculated to be secondary to septicemia or endotoxemia from the ulcerative gastritis. The uterus had suppurative, mild, diffuse, acute endometritis suspected to be secondary to immunosuppression.

Immunohistochemical detection of BVDV antigen identified numerous skin and pancreatic acinar epithelial cells that had strongly positive cytoplasmic immunoreactivity. Parotid salivary gland acinar and ductal epithelial cells comprised approximately

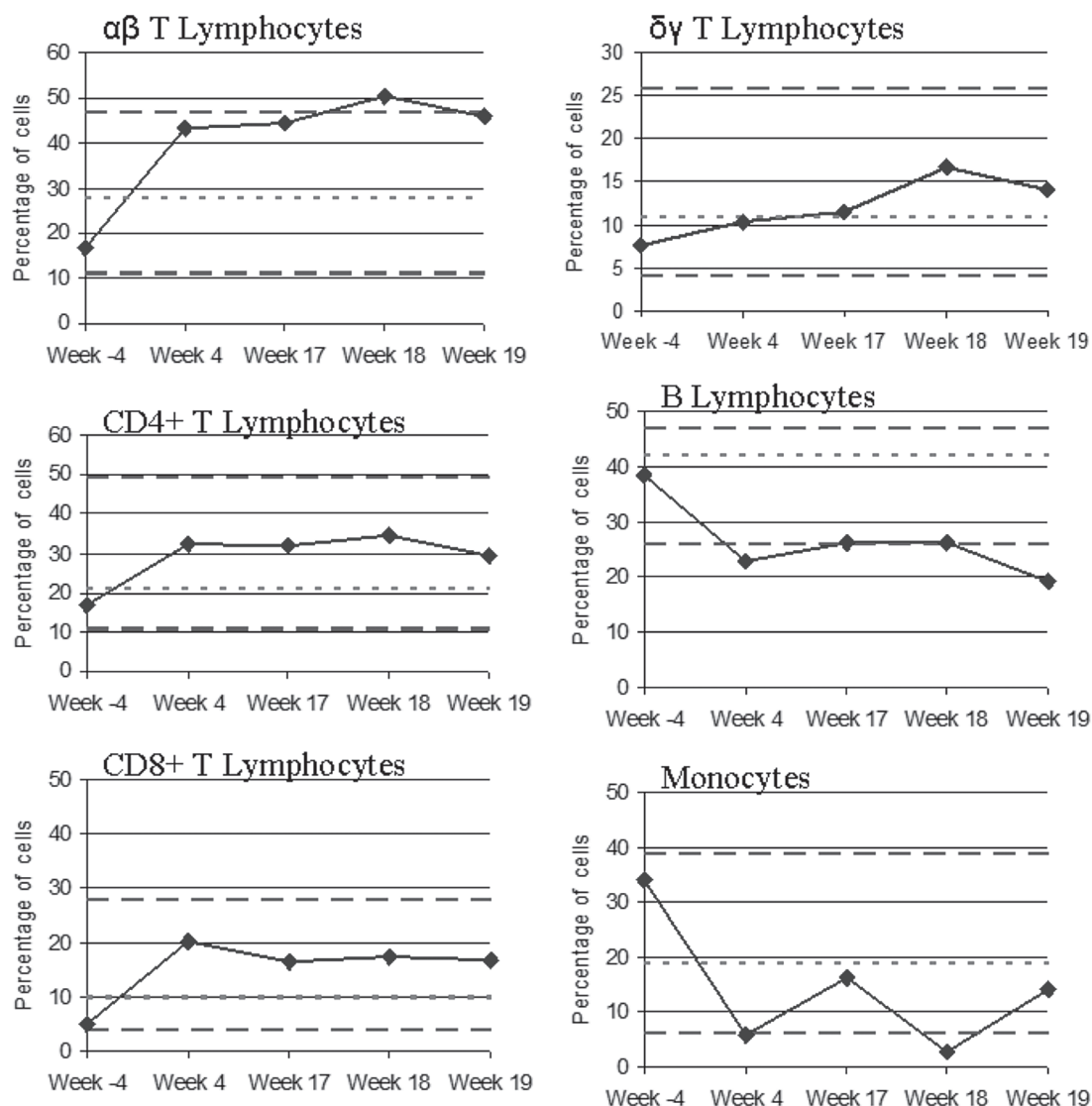


Figure 2. Mean flow cytometric results from previously unexposed alpacas exposed to BVDV persistently infected alpacas.

Values represent percentage of peripheral blood mononuclear cell populations. Samples from "Week -4" were collected prior to introduction to PI alpacas. Samples from "Week 17," "Week 18," and "Week 19" were collected immediately prior to reintroduction, at 5 d of re-exposure, and at 14 d of re-exposure to the PI alpacas, respectively. Dashed lines (---) represent upper and lower normal ranges and dotted lines (.....) represent the mean normal value obtained from 33 healthy camelids (1- to 11-months-old). Normal values provided by WSU Monoclonal Antibody Center.

5% of the section, and were stippled with strongly positive cytoplasmic immunoreactivity. The PCR detected BVDV on tracheal and nasal exudate swabs, whole blood and serum, urine, feces, liver, lung, kidney, spleen, and thymus.

Discussion

Research on PI bovine calves has identified BVD virus in most tissues and viral shedding from multiple sites (39,40). Previous reports on 5 confirmed PI alpacas identified virus in various organs including gastrointestinal tract, testes, prostate, kidney, and parotid salivary gland (11,12,14,16). Identification of virus in these organs suggests potential routes for viral shedding. In the present study, viral shedding was documented from urine, nasal, and oral swabs from both PI alpacas and from feces from

1 PI alpaca. The negative results from swabs obtained from WSU-S at 11 mo could have been due to improper collection, storage, or processing or may represent a true negative period of shedding. Reports have identified intermittent viremia in PI cattle suggesting diminished or variable BVDV shedding; however, there are no similar reports in alpacas (21,41). Variations in IHC and PCR results on postmortem tissues from WSU-D were suspected to be due to variations in testing sensitivity.

Naïve alpacas became viremic between 3 and 5 d of direct or indirect exposure to the PI crias. Transmission of virus was suspected to be via aerosolized bodily secretions with infection likely through the oronasal mucus membrane contact since the indirect fence-line contact alpacas readily developed transient infections. Other potential exposure routes that could not be

ruled out included nose-to-nose contact through the fence, direct contact with bodily fluids, and spitting. During the experimental period, manure and urination areas were not located adjacent to the shared fence line and there was no evidence of spitting. Alpacas in pen 1 shared feed, water buckets, and a manure pile with the PI alpacas suggesting that both indirect and direct transmission were probable. Both housing situations mimicked that commonly seen on alpaca farms where either direct physical contact or indirect (adjacent, fence line) contact can occur.

After a presumed incubation period of 5 to 8 d, BVDV nucleic acid was detectable by PCR on whole blood samples for 10 to 13 d in previously BVDV-naïve alpacas. This finding is similar to that reported in cattle where acute BVDV infections have an incubation period of 5 to 7 d and viremia persists for up to 15 d (18,26). Interestingly, viral shedding was not detected in the oral or nasal secretions of the TI alpacas whereas TI cattle readily shed virus and are transmission sources for other cattle (41).

Following the initial 4-week exposure to the PI alpacas, the 6 exposed alpacas were transferred to a remote area where their BVDV serum antibody concentrations were measured every 2 wk. Average BVDV antibody concentrations peaked between 2 and 3 mo post initial exposure. Approximately 4 mo after initial exposure the 6 male alpacas were re-exposed to the remaining PI alpaca (WSU-S). Upon re-exposure, the antibody titers did not significantly change, and appeared to be protective, as all 6 remained PCR negative. Again, our findings are similar to those described with acute BVDV infections in cattle where detectable serum antibodies were present 2 to 3 wk post-exposure and peaked at 2 to 3 mo unless exposure to PI cattle was maintained (42,43).

Acute BVDV infections within endemic cattle herds are usually inapparent or subclinical. Previous reports and the results from this project, suggest a similar, mild clinical disease process in alpacas. Several animals from both pens developed mildly elevated temperatures and soft stools, and a smaller proportion developed mild diarrhea and excessive serous nasal discharge. Such mild clinical signs may be missed in production units unless close, daily monitoring of animals is practiced.

Bovine viral diarrhea virus has an immunosuppressive effect in both TI and PI cattle. Immunosuppression increases the likelihood of clinically significant secondary infections, especially when animals are introduced into new or stressful environments such as feedlots or sale yards (44). These alpacas were maintained in an isolation facility which limited the exposure of the TI alpacas to potential infectious agents during the acute infection period and signs of secondary infections were not observed. The PI alpacas experienced recurrent febrile periods with increased nasal discharge indicative of secondary infections that were responsive to antibiotic therapy. However, WSU-D continued to deteriorate and was humanely euthanized at 8 mo of age. Postmortem examination revealed an upper and lower respiratory tract infection, generalized lymphoid depletion, and evidence of disseminated BVDV infection.

In cattle, BVDV affects both the production and function of lymphocytes, monocytes, and neutrophils and alters

immune-related cytokines (26). Transiently-infected cattle typically experience lymphoid tissue depletion, leukopenia, and lymphopenia (45,46). Similar decreases were not detected in the transiently infected alpacas and lymphoid biopsies were not obtained; therefore, the existence of lymphoid depletion could not be determined. Mildly decreased hematocrits were detected in the majority of transiently infected alpacas, and the condition resolved over time without treatment. The transient anemia may have resulted from temporary, viral-induced, suppression of bone marrow activity; however, similar effects have not been reported in cattle.

Flow cytometric analysis of peripheral leukocyte populations from BVDV TI or PI alpacas has not been previously published; therefore, results should not be over interpreted. Results in PI alpacas were typically within normal limits of healthy alpacas, which is similar to results from cattle persistently infected with type 1 BVDV in which leukocyte populations did not significantly differ from those of BVDV-free animals (47). When comparing cell populations before and after initial exposure to the PI alpacas, all T lymphocyte subsets increased and B lymphocytes and monocytes populations decreased. Monocyte populations fluctuated considerably over both exposure periods. Changes during the second exposure period were variable. In contrast, acutely infected cattle have decreased proportions of CD8+ and CD4+ T lymphocytes, variable changes in B lymphocytes, and no significant variation in monocytes or $\gamma\delta$ T lymphocytes (46,48). Due to the sampling time frame for the initial exposure trial, transient changes in alpaca cell populations may not have been appreciated and the values observed may represent a rebound effect or normal age related fluctuations. The various cell populations did not return to pre-exposure levels prior to the second exposure period and except for monocytes, remained relatively constant following the initial exposure. These variations may indicate differences in the alpaca immunological response to the virus versus that observed in cattle or may be normal variations occurring over time in juvenile alpacas. Further testing is needed to compare BVDV transiently infected and uninfected alpacas over time to better explain the variation observed here.

Results of the present study suggest that the most likely route of BVDV infection in alpacas is via the oronasal mucosa, presumably after inhalation of viral particles present in the bodily secretions of infected animals. Mild clinical signs observed during acute, transient infections in alpacas suggest that infections may be inapparent to owners and producers. While the animals evaluated in this project appeared to resist subsequent infection upon secondary re-exposure to viral-shedding PI animals, the duration of this potentially protective effect was not determined. The apparent lack of shedding by transiently infected alpacas may contribute to the relatively low prevalence of clinically evident BVDV infection in alpacas as well as the low prevalence of persistent infections in alpacas compared with cattle.

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